IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of : Yasuhiro Sakai et al.
Serial No. : 10/005,753
Filed : October 29, 2001
For : Method of Staining, and Detecting and Counting Bacteria, and a Diluent for Bacterial Stain

DECLARATION

I, Ryoko KOBAYASHI, declare that I am acquainted with both the Japanese and English languages, that the English translation attached hereto is a true and accurate translation of Japanese Patent Application No. 2000-334641.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed at Osaka, Japan on this 26th day of October 2004

R. Kobayashi

Ryoko KOBAYASHI

PATENT OFFICE
JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this office.

Date of Application: November 1, 2000

Application Number: Patent Application
No. 2000-334641

Applicant: SYSMEX CORPORATION

Date: December 14, 2001

Commissioner,
Patent Office Kozo OIKAWA

[Document Name] Application for Patent
[Sorting Number] 00-044JP
[Filing Date] November 1, 2000
[Addressee] The Commissioner of the Patent Office
[International Patent Classification] G01N 33/48
[Inventor]
[Address] c/o SYSMEX CORPORATION
5-1, Wakinohama-Kaigandori 1-chome, Chuo-ku, Kobe-shi, Hyogo Japan
[Name] Yasuhiro SAKAI
[Inventor]
[Address] c/o SYSMEX CORPORATION
5-1, Wakinohama-Kaigandori 1-chome, Chuo-ku, Kobe-shi, Hyogo Japan
[Name] Yasuyuki KAWASHIMA
[Inventor]
[Address] c/o SYSMEX CORPORATION
5-1, Wakinohama-Kaigandori 1-chome, Chuo-ku, Kobe-shi, Hyogo Japan
[Name] Junya INOUE
[Inventor]
[Address] c/o SYSMEX CORPORATION
5-1, Wakinohama-Kaigandori 1-chome, Chuo-ku, Kobe-shi, Hyogo Japan
[Name] Yoshiro IKEUCHI
[Applicant]
[Identification Number] 390014960
[Name] SYSMEX CORPORATION
[Attorney]
[Identification Number] 100088867
[Patent Attorney]
[Name] Tadatsugu NISHINO
[Indication of Fee]
[Payment] Prepayment
[Prepayment Register Number] 059617
[Amount] 21000
[Item of attached Documents]

[Name of Item] Specification 1
[Name of Item] Drawing 1
[Name of Item] Abstract of the Disclosure 1
[Number of the General Power of Attorney] 9723350
[Proof] Required

[NAME OF THE DOCUMENT] Specification

[TITLE OF THE INVENTION] Method of Staining Bacteria

[CLAIMS]

[Claim 1] A method of staining bacteria comprising: working a polymethine dye on a sample in the presence of a substance capable of reducing nitrite ions to stain bacteria in the sample.

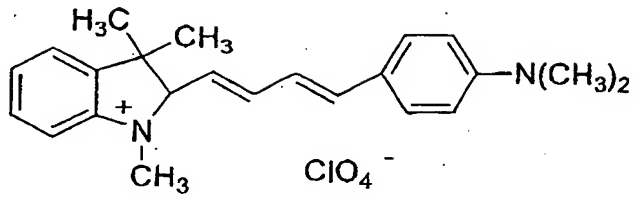
[Claim 2] A method of staining bacteria according to claim 1, wherein the substance capable of reducing nitrite ions is selected from the group consisting of: ascorbic acid or its salt, isoascorbic acid or its salt, sulfamic acid, sulfanilic acid, sulfanilamide, aminomethane, aminomethanesulfonic acid, aminoethanesulfonic acid, glycine, glutamic acid, glutamine, asparatic acid, asparagine, methionine, glutathione, cysteine, mercaptoethanol, mercaptoacetic acid, thiophenol, 3-mercaptopropionic acid, sodium sulfite, sodium pyrosulfite, hydroxylamine hydrochloride, sodium phosphinate and urea.

[Claim 3] A method of staining bacteria according to claim 1, wherein the polymethine dye is at least one selected from the following group consisting of:

(1) Thiazole Orange;

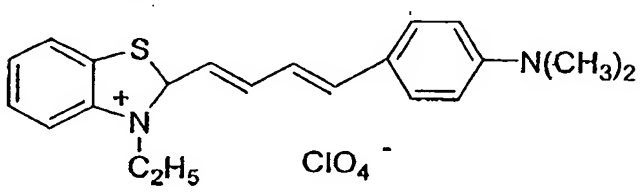
(2)

[Chemical 1]



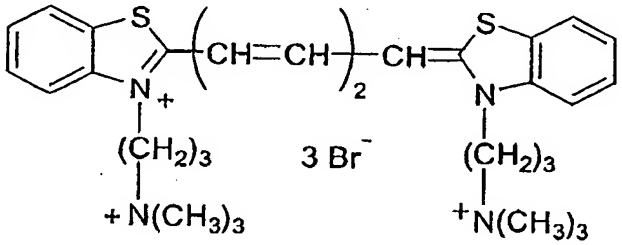
(3)

[Chemical 2]



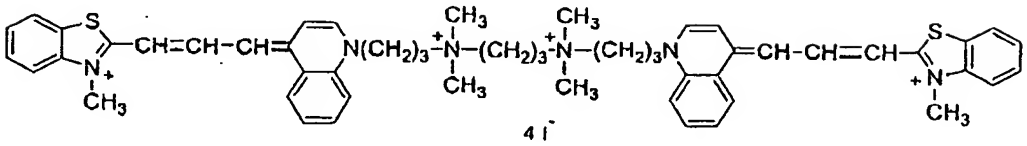
(4)

[Chemical 3]



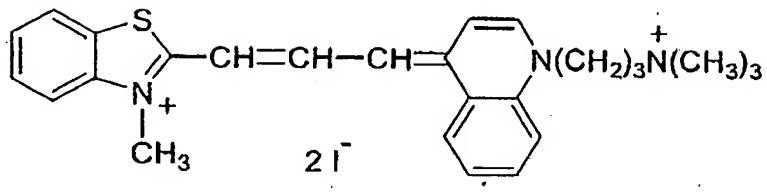
(5)

[Chemical 4]



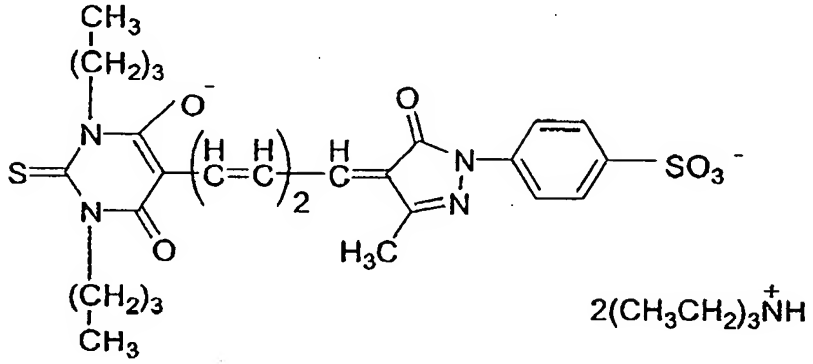
(6)

[Chemical 5]



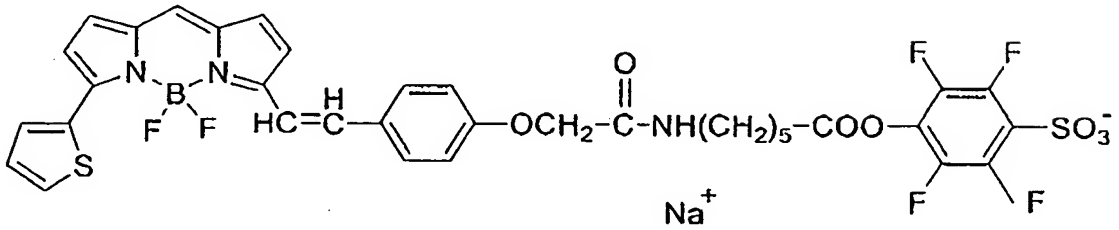
(7)

[Chemical 6]



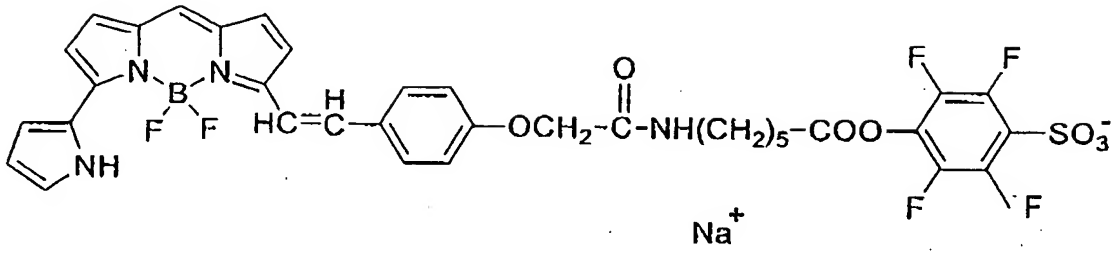
(8)

[Chemical 7]



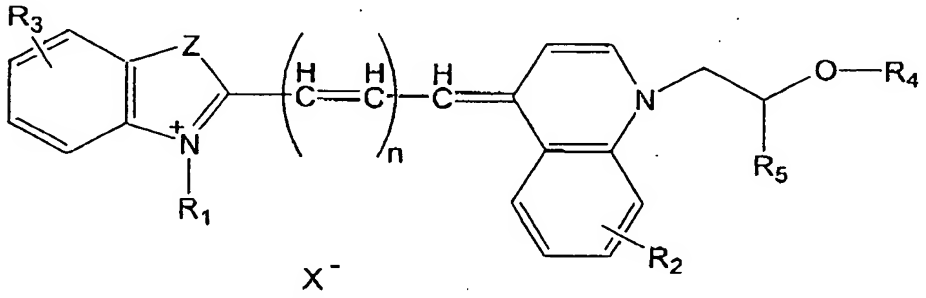
(9)

[Chemical 8]



(10) a compound represented by the following general formula:

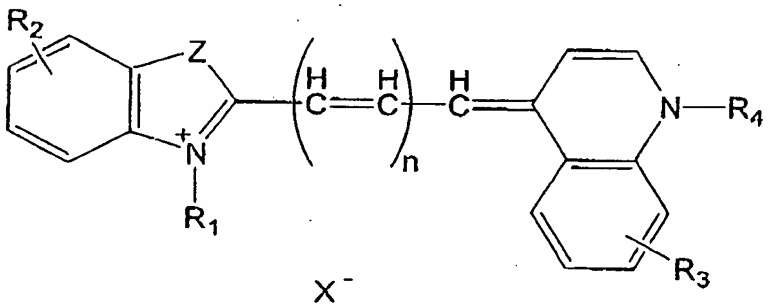
[Chemical 9]



wherein R_1 is a hydrogen atom or a C_{1-3} alkyl group; R_2 and R_3 are a hydrogen atom, a C_{1-3} alkyl group or a C_{1-3} alkoxy group; R_4 is a hydrogen atom, an acyl group or a C_{1-3} alkyl group; R_5 is a hydrogen atom or a C_{1-3} alkyl group which may be substituted; Z is a sulfur atom, an oxygen atom or a carbon atom substituted with a C_{1-3} alkyl group; n is 1 or 2; X^- is an anion; and

(11) a compound represented by the following general formula:

[Chemical 10]



wherein R_1 is a hydrogen atom or a C_{1-18} alkyl group; R_2 and R_3 are a hydrogen atom, a C_{1-3} alkyl group or a C_{1-3} alkoxy group; R_4 is a hydrogen atom, an acyl group or a C_{1-18} alkyl group; Z is sulfur, oxygen or carbon having a C_{1-3} alkyl group; n is 0, 1 or 2; X^- is an anion.

[DETAILED DESCRIPTION OF THE INVENTION]

[0001]

[Technical Field of the Invention]

The present invention relates to a method of staining bacteria in clinical samples, in particularly preferably, bacteria in urine samples.

[0002]

[Prior Art]

The number of bacteria in urine is an important parameter in clinical diagnosis to judge the presence of infection. In general, the presence of bacteria of 10^5 or more/ml in urine is recognized as a criterion of positive urinary tract infection. If urine contains bacteria of 10^3 or less/ml, it is diagnosed as contaminated urine (normal bacteria flora), i.e., negative urinary tract infection. If bacteria of about 10^4/ml is observed, the diagnosis is reserved but the sample is often re-examined.

[0003]

Conventionally, observation of bacteria in urine has been performed by microscopic examination of Gram stained bacteria, unstained bacteria without Gram staining treatment or

fluorescence-stained bacteria.

[0004]

Urine often contains contaminants such as mucus threads, crystals, amorphous salts and cell fragments that are clinically insignificant. These substances hinder the measurement of significant particles (in particular bacteria) so that it has been difficult to accurately count the number of bacteria. Actually, there has been no method of counting bacteria of about 10^4/ml, accurately.

[0005]

In the case of Gram stain, bacteria and contaminants are stained simultaneously so that counting loss of bacteria of a small number occurs frequently in the microscopic examination. Further, Gram stain includes a number of staining steps and takes time (about 15 minutes) so that working efficiency is poor.

[0006]

The microscopic examination of bacteria without staining treatment can be carried out quickly, but it cannot discriminate bacteria particularly when coccus contaminants are contained.

[0007]

The microscopic examination of fluorescence-stained bacteria shows better detectability than the above-mentioned two methods. However, there has not been established how to eliminate other contaminants than bacteria and to stain the bacteria quickly.

[0008]

Agar medium method, which is a standard method, requires 16

hours or more to determine the bacteria number, so that it cannot be regarded as a quick method.

[0009]

USP 4,622,298, and Japanese Unexamined Patent Publication No. Hei 9 (1997)-119926 and No. Hei 9 (1997)-329596 each proposes a method of detecting bacteria in a fluorescence-stained urine sample with a flow cytometer. A polymethine dye utilizes for fluorescence staining.

[0010]

[Problems that the Invention is to Solve]

However, when a polymethine dye is used for staining bacteria, some bacteria are not sufficiently stained. For example, in the case of a sample in which nitrate-reducing bacteria proliferate and produce a large amount of nitrite, nitrite ions decompose the polymethine dye so that the dye does not effectively work on the bacteria staining.

[0011]

Usually, bacteria are stained well at acidic pH. Further, a urine sample which contains mucus threads is effective in the bacteria staining. However, effect of the nitrite ions is promoted at acidic pH.

[0012]

An object of the present invention is to provide a method of staining bacteria which allows quick and efficient detection of bacteria even if a sample contains nitrite ions at high concentration.

[0013]

[Means for Solving the Problems]

A method of staining bacteria of the present invention comprises working a polymethine dye on a sample in the presence of a substance capable of reducing nitrite ions to stain bacteria in the sample.

[0014]

[Mode for Carrying Out the Invention]

In the present invention, the sample is not particularly limited as long as it contains bacteria, however, it is particularly effective for a urine sample.

[0015]

The substance capable of reducing nitrite ions may be one or more kinds selected from the group comprising of: ascorbic acid or its salt, isoascorbic acid or its salt, sulfamic acid, sulfanilic acid, sulfanilamide, aminomethane, aminomethanesulfonic acid, aminoethanesulfonic acid, glycine, glutamic acid, glutamine, asparatic acid, asparagine, methionine, glutathione, cysteine, mercaptoethanol, mercaptoacetic acid, thiophenol, 3-mercaptopropionic acid, sodium sulfite, sodium pyrosulfite, hydroxylamine hydrochloride, sodium phosphinate and urea. With respect to the concentration thereof, it may be contained at 10 mM or more in a diluent for diluting the sample. Preferably, ascorbic acid may be contained at 85 to 115 mM, sulfamic acid may be contained at 40 to 200 mM, cysteine, glutathione, sodium sulfite may be contained at 10 to 50 mM and urea may be contained at 0.5 M or more, respectively. Higher concentration of urea is not preferred because it may cause denaturation of cells. In

general, it is considered that 0.06 mg/ml of nitrite ions are produced in the presence of nitrate-reducing bacteria of 10^5/ml. Further, proliferation of bacteria is considered to be limited up to 10^8 to 10^9/ml and it does not (cannot) proceed any higher. Therefore, the substance may be used in such an amount that it can reduce nitrite ions produced by bacteria of 10^5 to 10^8/ml.

[0016]

In the present invention, pH at the staining step is not specifically limited as long as it allows the bacteria staining. Where a urine sample is stained at an acidic pH, (1) bacteria is stained better than in a neutral or alkaline state and (2) nonspecific staining of mucus threads is prevented and the mucus threads is lysed to a certain extent. Thus, the acidic state is advantageous to the bacteria staining.

[0017]

A buffer of pKa 1 to 5 can be used to maintain the acidic state. The buffer is not particularly limited, but a compound capable of maintaining pH 2.0-3.0 may be used. As the buffer, it may be utilized citric acid salts, phosphoric acid salts, phthalic acid salts, glycine, succinic acid, lactic acid, β-alanine, ε-aminocaproic acid, fumaric acid and the like. Appropriate use amount thereof is such that the above-mentioned pH range is maintained, preferably about 10 to 500 mM in the sample.

[0018]

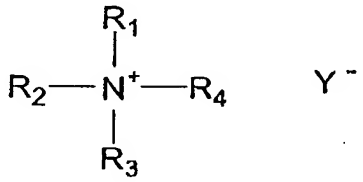
A cationic surfactant may be preferably added to the sample

containing bacteria because the cell membrane of the bacteria may be damaged so that a dye enters cells easily. As a result, substances in bacterial cells are effectively bound to the dye and bacteria are stained sufficiently, therefore bacterial cells are easily discriminated from contaminants. On the other hand, mucous fibers, erythrocytes, cell fractures and others are dissolved/shrunk so that their effects on detection of bacteria are reduced.

[0019]

No particular limitation is given to the cationic surfactant, but preferably is used a quarternary ammonium salt represented by the following formula:

[Chemical 11]



wherein R_1 is a C_{8-18} alkyl group; R_2, R_3 and R_4, the same or different, are a C_{1-3} alkyl group or a benzyl group; Y^- is a halogen ion.

[0020]

For example, are suitably used decyl trimethyl ammonium salts, dodecyl trimethyl ammonium salts, tetradecyl trimethyl ammonium salts, hexadecyl trimethyl ammonium salts, octadecyl trimethyl ammonium salts and the like. The concentration of the cationic surfactant may be 10 to 30000 mg/ml, preferably 100 to 3000 mg/ml.

[0021]

The dye is not particularly limited as long as it can stain bacteria. Where a urine sample is examined, a dye capable of staining bacteria under an acidic state is preferably used. The concentration thereof may suitably be determined depending on the kind of dye, for example, in the range of 0.1 to 100 ppm (final concentration). In view of bacteria detectability, a fluorescent dye which is at least bonded to one of components constituting bacteria and emits fluorescent light is advantageously used. From this point of view, polymethine dyes are preferable. For example, the following dyes (1) to (11) are used:

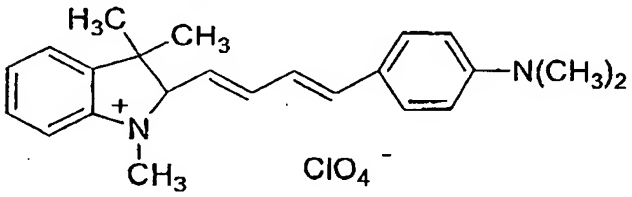
[0022]

(1) Thiazole Orange;

[0023]

(2);

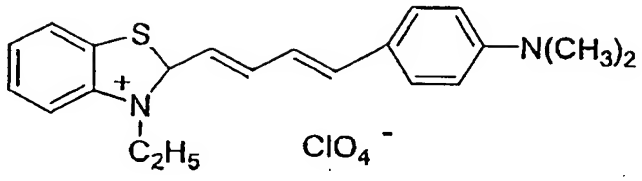
[Chemical 12]



[0024]

(3);

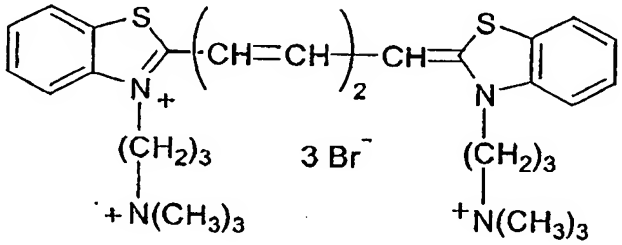
[Chemical 13]



[0025]

(4);

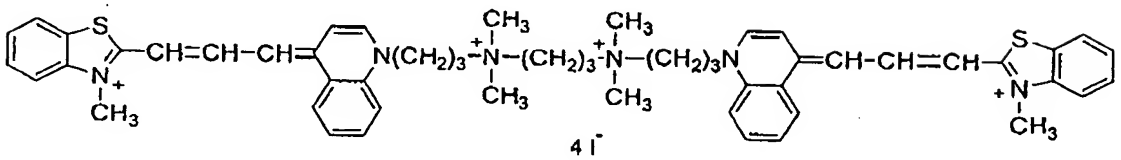
[Chemical 14]



[0026]

(5);

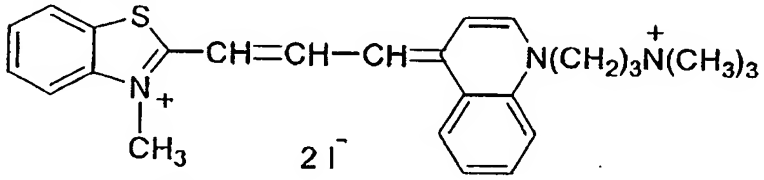
[Chemical 15]



[0027]

(6);

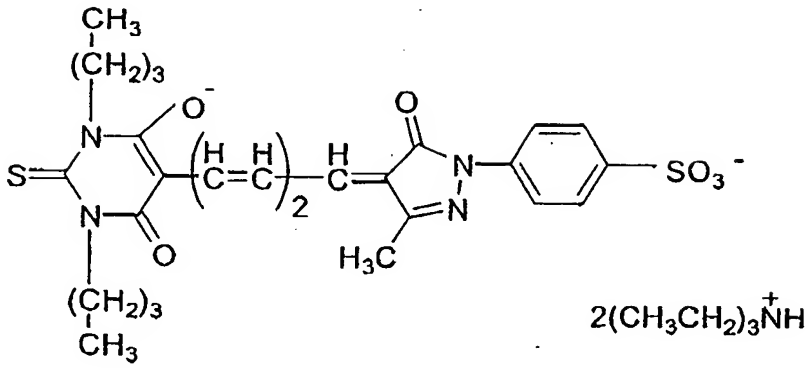
[Chemical 16]



[0028]

(7);

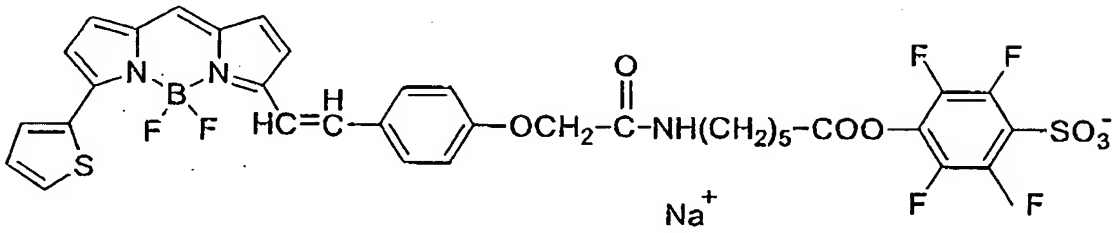
[Chemical 17]



[0029]

(8);

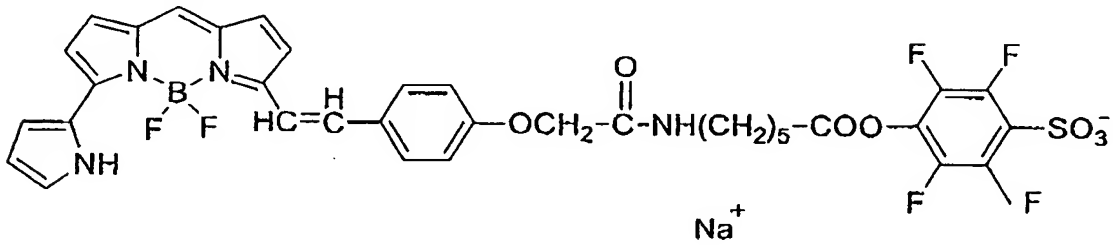
[Chemical 18]



[0030]

(9);

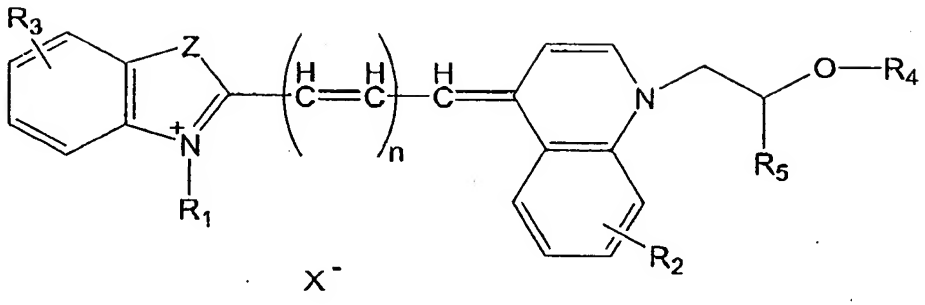
[Chemical 19]



[0031]

(10) a compound represented by the following general formula:

[Chemical 20]

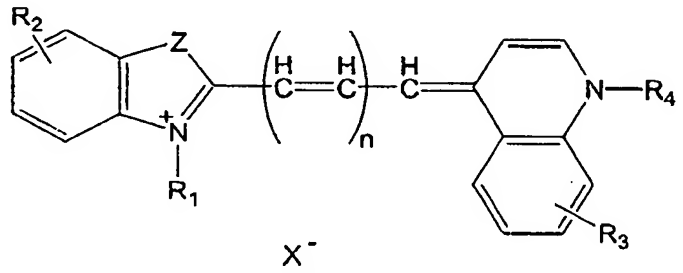


wherein R_1 is a hydrogen atom or a C_{1-3} alkyl group; R_2 and R_3 are a hydrogen atom, a C_{1-3} alkyl group or a C_{1-3} alkoxy group; R_4 is a hydrogen atom, an acyl group or a C_{1-3} alkyl group; R_5 is a hydrogen atom or a C_{1-3} alkyl group which may be substituted; Z is a sulfur atom, an oxygen atom or a carbon atom substituted with a C_{1-3} alkyl group; n is 1 or 2; X^- is an anion; and

[0032]

(11) a compound represented by the following general formula:

[Chemical 21]



wherein R_1 is a hydrogen atom or a C_{1-18} alkyl group; R_2 and R_3 are a hydrogen atom, a C_{1-3} alkyl group or a C_{1-3} alkoxy group; R_4 is a hydrogen atom, an acyl group or a C_{1-18} alkyl group; Z is sulfur, oxygen or a carbon atom substituted with a C_{1-3} alkyl group; n is 0, 1

or 2; X^- is an anion.

[0033]

Among the above-mentioned dyes, (1) is commercially available. (2) and (3) are supplied by Nippon Photosensitive Dye Laboratory Ltd., and (5) to (9) are supplied by Molecular Probes, Inc.

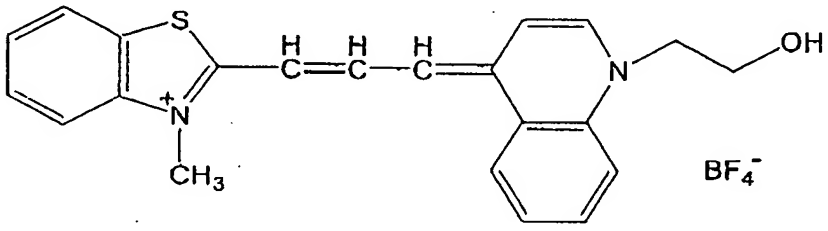
[0034]

Manufacturing methods of (10) and (11) are described in Japanese Unexamined Patent Publications Nos. Hei 9(1997)-104683 and Hei 10(1998)-319010, respectively.

[0035]

Among the dyes (10), a dye represented by the formula:

[Chemical 22]

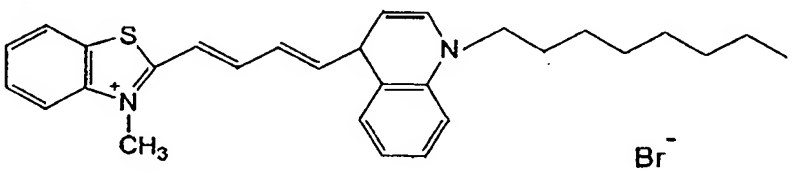


is particularly suitable.

[0036]

Further, among the dyes (11), a dye represented by the formula:

[Chemical 23]



is particularly suitable.

[0037]

Further, where a urine sample is examined, the staining is carried out by further utilizing an inorganic salt of either sulfate or nitrate. This is preferable since fluorescent dye transmissivity of bacteria is enhanced and nonspecific staining of contaminants is prevented. The inorganic salt may be used in a concentration of about 10 to 500 mM, preferably about 50 to 200 mM in the sample.

[0038]

By employing the method of staining bacteria of the present invention, a sample containing high amount of bacteria which reduce nitrite and produce nitrous acid, e.g., intestinal bacteria such as Staphyrococcus aurcus, Gram-negative facultative bacilli such as E. coli, Klebsiella sp. and Proteus sp., can be suitably stained. As a sample, not only urine sample, but also a clinical sample such as blood, spinal fluid or the like may be used.

[0039]

The staining method of the present invention may be carried out by mixing the sample, an aqueous solution containing the substance capable of reducing nitrite ions and a solution containing the dye. The dye may be contained in the aqueous solution containing the substance capable of reducing nitrite ions. However, where the dye to be utilized is unstable in the aqueous solution, it may be dissolved in a water-soluble organic solvent such as methanol, ethanol or ethylene glycol and then mixed for use with the aqueous

solution containing the substance capable of reducing nitrite ions. This improves storage stability of the dye.

[0040]

Temperature and time for the staining are not particularly limited, but the staining may be performed at a temperature of 15 to 50℃ for 15 minutes immediately after the mixing.

[0041]

The sample stained by the method of the present invention may be observed with a microscope or an imaging apparatus to detect bacteria. Alternatively, bacteria can be detected and counted by using a flow cytometer with high accuracy.

[0042]

That is, bacteria can be detected by:

(1) diluting a sample containing bacteria with an aqueous solution containing a substance capable of reducing nitrite ions,

(2) treating the above sample with a polymethine fluorescent dye for a predetermined time for staining reaction,

(3) introducing the thus treated sample into a detecting part of a flow cytometer and irradiating cells of the stained bacteria one by one with light to measure scattered light and fluorescent light emitted from each of the cells; and

(4) discriminating the bacteria from other components in accordance with an intensity of a scattered light signal and an intensity of a fluorescent light signal or a pulse width reflecting the length of particles to count the bacteria.

[0043]

Particularly, when using a urine sample in which various other components are present than bacteria, discrimination of bacteria from other components and counting of bacteria are carried out in accordance with combination of signals obtained by using a measurement. Example of the combination of signals includes, for example, a forward scattered light intensity and a forward scattered light pulse width, a forward scattered light intensity and a fluorescent light intensity, a forward scattered light pulse width and a fluorescent light intensity, and the like. In a suitable manner, for example, firstly, a scattergram is formed from the combination of the forward scattered light intensity and the forward scattered light pulse width, and then gating is performed to a mass including bacteria specified on the scattergram to separate mucus threads, mainly. Further, another scattergram is formed from the forward scattered light intensity and the fluorescent light intensity of the gated mass to separate bacteria from other components (crystals, cell fragments and the like) based on the difference in the fluorescent light intensity. The outline of the method is shown in Fig. 4. Where the sample contains bacteria only, a scattergram is formed from the forward scattered light intensity and the fluorescent light intensity to count them.

[0044]

[Examples]

Hereinafter, preferred examples of the method of staining bacteria according to the present invention are described, but the

present invention is not limited thereto.

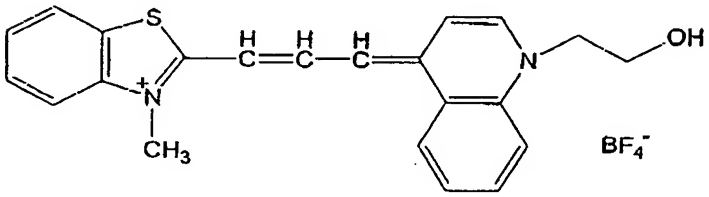
Example 1

Reagent Composition

(Diluent)

Citric acid	92.3 mM
Sodium hydroxide	0.75 g/l (up to pH 2.5)
Tetradecyl trimethyl ammonium bromide	0.1 %(w/v)
Sodium sulfate	90 mM
Ascorbic acid	85 mM
(Staining solution)	
Dye A (of the following structural formula)	40 ppm (in ethylene glycol)

[Chemical 24]



[0045]

To 140 µl of a sample containing a large amount of nitrite ions (bacteria concentration of 5.0×10^6/ml; hospital urine), 952 µl of the above-mentioned diluent was added and the staining solution was added so that the final concentration of the dye A would be 1 ppm. Staining was carried out at 40℃ for 20 seconds and then scattered light and fluorescent light were measured by a flow cytometer provided with a red semiconductor laser as a light source (amount of examined urine: 8.0 µl). Then, as shown in Fig. 1, a scattergram was formed

with a fluorescent light intensity (FLI) as an horizontal axis and a forward scattered light intensity (FSLI) as a vertical axis. As a control, measurement was performed using a reagent containing no ascorbic acid (Fig. 2).

[0046]

In the case where the reagent without ascorbic acid was used, bacteria were not stained and the fluorescent light intensity was zero. In contrast, bacteria were stained and detected when ascorbic acid was added.

[0047]

Example 2

Measurement was performed in the same manner as in Example 1 except that sulfamic acid of 100 mM was used instead of ascorbic acid in the diluent. Fig. 3 shows the results. Bacteria were stained and detected as in Example 1.

[0048]

[Effect of the Invention]

According to the method of staining bacteria of the present invention, the substance capable of reducing nitrite ions is added. Therefore, even if nitrate-reducing bacteria produce nitrite ions in the sample, bacteria can be quickly detected without an influence therefrom.

[Brief Description of the Drawings]

[Fig. 1]

Fig. 1 is a scattergram of a fluorescent light intensity – a

forward scattered light intensity obtained in the case where ascorbic acid is used as a reducing agent in Example 1 of the present invention.

[Fig. 2]

Fig. 2 is a scattergram of a fluorescent light intensity – a forward scattered light intensity obtained in the case where the reducing agent is not used in Example 1 of the present invention.

[Fig. 3]

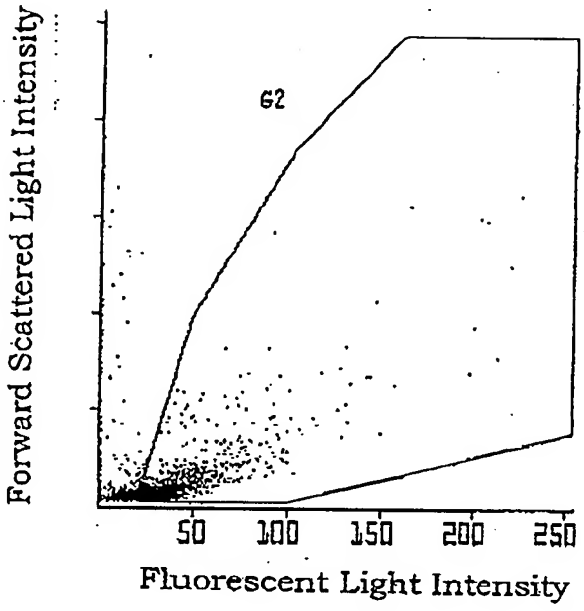
Fig. 3 is a scattergram of a fluorescent light intensity – a forward scattered light intensity obtained in the case where sulfamic acid is used as the reducing agent in Example 2 of the present invention.

[Fig. 4]

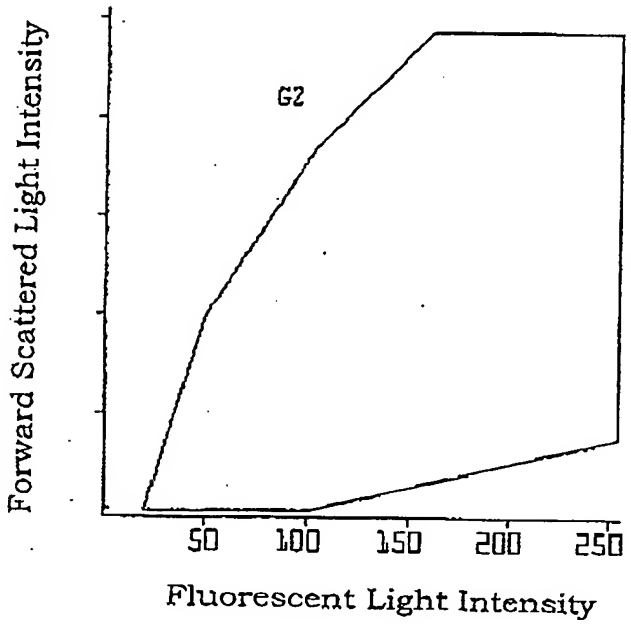
Fig. 4 is a view illustrating the outline of a method of discriminating and counting bacteria after carrying out the method of detecting bacteria according to the present invention.

[NAME OF THE DOCUMENT] Drawings

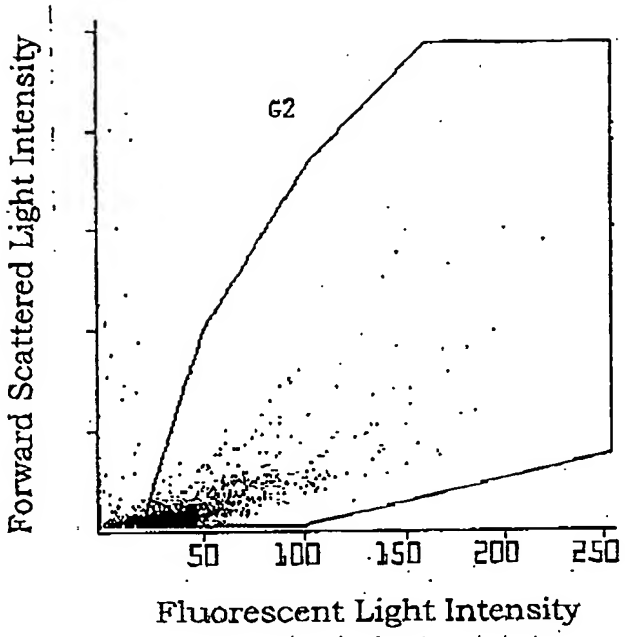
[Fig.1]



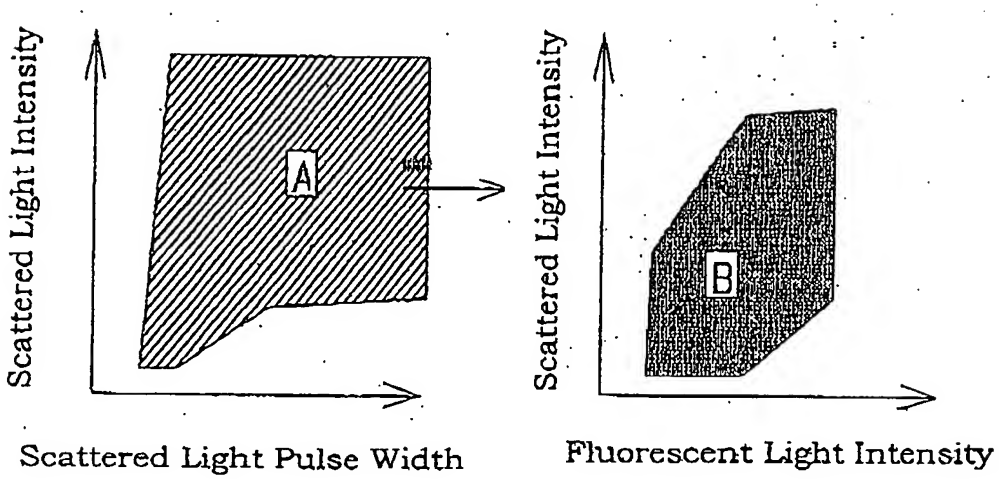
[Fig. 2]



[Fig. 3]


Forward Scattered Light Intensity
G2
50
100
150
200
250
Fluorescent Light Intensity

[Fig. 4]


Scattered Light Intensity
A
Scattered Light Pulse Width
Scattered Light Intensity
B
Fluorescent Light Intensity

[NAME OF THE DOCUMENT] Abstract

[ABSTRACT]

[OBJECT] To provide a staining method which allows quick and efficient detection of bacteria even if a sample contains nitrite ions at high concentration.

[MEANS TO ATTAIN OBJECT] Bacteria are stained by working a polymethine dye on a sample in the presence of a substance capable of reducing nitrite ions.

[SELECTED FIGURE] Fig. 1